Construction and characterization of an infectious cDNA clone of rat hepatitis E virus

Tian-Cheng Li,1 Tingting Yang,2 Sayaka Yoshizaki,1 Yasushi Ami,3 Yuriko Suzuki,3 Koji Ishii,1 Kei Haga,1 Tomofumi Nakamura,1 Susumu Ochiai,4 Wakita Takaji1 and Reimar Johne5

1Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-murayama, Tokyo 208-0011, Japan
2Department of Clinical Laboratory, Affiliated Hospital of Qingdao University Medical College, Jiangsu Road 16, Qingdao 266003, PR China
3Division of Experimental Animals Research, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-murayama, Tokyo 208-0011, Japan
4Research and Production Technology Department, The Research Foundation for Microbial Diseases of Osaka University, 5-34-4 Kumegawacho Higashimurayama-shi, Tokyo 189-0003, Japan
5Department Biological Safety, Federal Institute for Risk Assessment, Max-Dohrn-Straße 8–10, 10589 Berlin, Germany

Rat hepatitis E virus (HEV) is related to human HEV and has been detected in wild rats worldwide. Here, the complete genome of rat HEV strain R63/DEU/2009 was cloned downstream of the T7 RNA polymerase promoter and capped genomic RNA generated by in vitro transcription was injected into nude rats. Rat HEV RNA could be detected in serum and faeces of rats injected intrahepatically, but not in those injected intravenously. Rat HEV RNA-positive faecal suspension was intravenously inoculated into nude rats and Wistar rats leading to rat HEV RNA detection in serum and faeces of nude rats, and to seroconversion in Wistar rats. In addition, rat HEV was isolated in PLC/PRF/5 cells from the rat HEV RNA-positive faecal suspension of nude rats and then passaged. The cell culture supernatant was infectious for nude rats. Genome analysis identified nine point mutations of the cell-culture-passaged virus in comparison with the originally cloned rat HEV genome. The results indicated that infectious rat HEV could be generated from the cDNA clone. As rats are widely used and well-characterized laboratory animals, studies on genetically engineered rat HEV may provide novel insights into organ tropism, replication and excretion kinetics as well as immunological changes induced by hepeviruses.

INTRODUCTION

Rat hepatitis E virus (HEV) was first identified in 2010 in faeces of wild rats from Germany (Johne et al., 2010a). The virus has since been detected in animals from several countries of Europe, Asia and in the USA (Johne et al., 2014a). The host species are mainly rat species (Rattus norvegicus, Rattus rattus and others), but rat HEV sequences have also been detected in the greater bandicoot (Bandicota indica) and the Asian musk shrew (Suncus murinus) (Guan et al., 2013; Li et al., 2013a; Johne et al., 2014a).

Analysis of the complete genome sequence indicated that rat HEV is a member of the family Hepeviridae, with a distant relationship to the human pathogenic HEV genotypes 1–4 (Johne et al., 2010b). Phylogenetic analysis indicated that rat HEV is most closely related to the recently identified ferret HEV (Raj et al., 2012); both viruses constitute a group clustering between human HEV genotypes 1–4 and hepeviruses from bats and birds (Johne et al., 2014a; Smith et al., 2014). All members of the family Hepeviridae have a genome of ssRNA with positive polarity and a length of ~7 kb, which is capped at the 5’ end and polyadenylated at the 3’ end. The rat HEV genome contains ORF1, 2 and 3 encoding a non-structural polyprotein, the capsid protein and a small phosphoprotein, respectively, which are present in all HEV-related viruses. In addition, the rat HEV genome has a small ORF4 overlapping with the 5’ region of ORF1, with unknown function (Johne et al., 2010b; Mulyanto et al., 2014).
Analysis of the amount of rat HEV RNA in organs of wild rats indicated a liver tropism of the virus (Johne et al., 2010b). The hepatotropism of rat HEV was also confirmed with laboratory rats intravenously inoculated with rat HEV-containing organ homogenates (Li et al., 2013b). Immunodeficient nude rats inoculated with rat HEV developed persistent infections with prolonged shedding of high amounts of virus (Li et al., 2013b). Experimentally infected Wistar rats showed transient virus shedding and developed anti-rat HEV-specific antibodies (Li et al., 2013b). Clinical signs were not recorded after experimental infection of rats (Li et al., 2013b; Purcell et al., 2011). Preliminary infection trials with rat-derived cell cultures did not result in rat HEV replication (Johne et al., 2010a). However, rat HEV was recently propagated successfully in the human hepatoma cell lines PLC/PRF/5, HuH-7 and HepG2 (Jirintai et al., 2014).

The zoonotic potential of rat HEV is not clear. Experimental infections of rhesus monkeys and pigs with rat HEV did not result in signs of virus replication (Cossaboom et al., 2012; Purcell et al., 2011). Serological analysis of human and porcine sera identified only a few sera showing antibodies with higher reactivity to rat HEV as compared with human HEV, indicating a very rare transmission of rat HEV-related viruses (Dremsek et al., 2012; Krumbholz et al., 2013).

Reverse genetics systems, which enable the generation of infectious virus from cloned cDNA, have been widely used for site-directed mutagenesis of viral RNA genomes following various applications in basic and applied virology (Hoenen et al., 2011; Stobart & Moore, 2014; Ye et al., 2014). A reverse genetics system for human HEV was first developed by Panda et al. (2000). In this system, the cDNA of the genome of a genotype 1 strain was cloned downstream of the T7 RNA polymerase promoter. The linearized cDNA clone was transcribed in vitro and thereafter transfected into cell cultures. The cell culture supernatant was shown to be infectious for rhesus monkeys; however, direct inoculation of the RNA into the monkeys did not result in generation of the virus. Emerson et al. (2001) showed that a capping step during in vitro transcription enabled the generation of infectious virus by intrahepatic inoculation into rhesus monkeys and chimpanzees. Similar systems have been developed for HEV genotypes 3 and 4, rabbit HEV, and avian HEV, some of them using transfection of RNA into cell cultures for the generation of infectious virus, others using direct inoculation into the liver of laboratory animals (Córdoba et al., 2012; Cossaboom et al., 2014; Huang et al., 2005a, b; Kwon et al., 2011; Yamada et al., 2009).

Here, in order to develop a reverse genetics system for rat HEV, its complete genome was cloned under control of the T7 RNA polymerase promoter. Capped in vitro transcribed rat HEV RNA was thereafter used to inoculate laboratory rats and the generation of infectious rat HEV was monitored. Generated virus was characterized by infectivity tests in rats and cell culture as well as by genome sequence analysis. The results should contribute to the development of a reliable reverse genetics system for rat HEV, enabling site-directed mutagenesis and subsequent phenotypic studies on the virus.

RESULTS

Construction of a genomic clone of rat HEV

The whole genome of the rat HEV prototype strain R63/DEU/2009 was amplified by reverse transcription (RT)-PCR in four overlapping fragments and cloned. The final genomic clone contained a T7 RNA polymerase promoter sequence at the 5’ end and a poly(A) sequence at the 3’ end of the rat HEV genome followed by a unique XbaI restriction site. A schematic map of the plasmid is shown in Fig. 1(a). Restriction analysis of the plasmid resulted in the expected DNA fragments (Fig. 1b). Sequencing of the cloned genome identified 23 point mutations as compared with the original sequence of strain R63/DEU/2009 (GenBank accession number GU345042). Most of the mutations were either synonymous or led to amino acids that have also been detected in other rat HEV strains at the respective positions (Table S1, available in the online Supplementary Material). Only four unique amino acid exchanges were detected, which were located exclusively within the hypervariable region of ORF1.

Recovery of rat HEV by inoculation of nude rats with in vitro transcribed RNA

The XbaI-linearized plasmid was transcribed in vitro, resulting in capped rat HEV genome-length RNA. A total of 500 μl in vitro transcribed RNA (2.6 × 10¹¹ copies μl⁻¹) was intrahepatically inoculated in two nude rats LR1 (6 weeks old, female) and LR2 (10 weeks old, male). In addition, two nude rats VR1 (6 weeks old, female) and VR2 (10 weeks old, male) were intravenously inoculated through the tail vein with the same dose of in vitro transcribed RNA. Rat HEV RNA was detected in the stool specimens from both LR1 and LR2 on day 14 post-inoculation (p.i.) at 4.65 × 10⁶ and 1.15 × 10⁸ copies g⁻¹, reaching a plateau on day 30 p.i. at 1.08 × 10¹¹ and 1.94 × 10¹¹ copies g⁻¹, respectively (Fig. 2). Rat HEV RNA was detected in sera of LR1 and LR2 on day 49 p.i. at 3.84 × 10⁷ and 4.75 × 10⁷ copies ml⁻¹, respectively. In contrast, rat HEV RNA could not be detected in stool specimens or serum of VR1 and VR2.

Demonstration of infectivity of rat HEV recovered from inoculated nude rats

Stool samples taken from LR1 and LR2 on day 30 p.i. were used to prepare sterile-filtered 10% suspensions. Two nude rats RR1 (13 weeks old, female) and RR2 (17 weeks old, male) and two 15-week-old female Wistar rats WR1 and WR2 were intravenously inoculated with 300 μl stool suspension from LR1 or LR2 (RR1 and WR1 inoculated
with suspension from LR1; RR2 and WR2 inoculated with suspension from LR2). Rat HEV RNA was detected in the stools of RR1 and RR2, starting on day 14 p.i. at 3.89 \(10^{10}\) and 4.73 \(10^{10}\) copies g\(^{-1}\), and thereafter at \(10^{10}\) copies g\(^{-1}\) (Fig. 3a). Rat HEV RNA was also detected in the sera of RR1 and RR2 at day 28 p.i. at 7.04 \(10^{7}\) and 1.11 \(10^{8}\) copies ml\(^{-1}\), respectively. In WR1 and WR2, anti-rat HEV IgG was detected beginning at day 14 p.i. and reached a plateau at day 28 p.i. (Fig. 3b). The alanine aminotransferase (ALT) levels of RR1, RR2, WR1 and WR2 were, 40 IU l\(^{-1}\) throughout the whole experiment.

**Isolation of rat HEV from inoculated nude rats in PLC/PRF/5 cells**

The sterile-filtered 10% rat stool suspension taken from LR1 on day 30 p.i. was inoculated onto PLC/PRF/5 cells. Rat HEV RNA was detected in PLC/PRF/5 cell culture supernatant on day 30 p.i. at 9.27 \(10^{6}\) copies ml\(^{-1}\) and reached a plateau on day 60 p.i. at 2.59 \(10^{9}\) copies ml\(^{-1}\). Thereafter, high amounts of rat HEV RNA were constantly detected \((>10^{9}\) copies ml\(^{-1}\)) until day 116 p.i. (Fig. 4a). The capsid protein of rat HEV was detected in culture supernatant beginning on day 44 p.i. and reached a plateau starting at day 64 p.i. until the end of the experiment (Fig. 4b). Inoculation of fresh PLC/PRF/5 cells with the cell culture supernatant taken at day 68 p.i. resulted in detection of rat HEV RNA as early as day 16 p.i. and capsid protein at day 24 p.i. In addition, the amounts of rat HEV RNA reached \((1.87 \times 10^{10})\) were considerably higher compared with the first passage of the virus (Fig. 4a, b). No cytopathic effect was observed during the whole experiment.

**Demonstration of infectivity of cell culture-derived rat HEV for nude rats**

Two 20-week-old female nude rats CR1 and CR2 were each intravenously inoculated with 0.5 ml cell culture supernatant from day 68 p.i. of the first passage. Rat HEV RNA was detected in stools beginning at day 12 p.i. at 1.35 \(10^{7}\) and 9.97 \(10^{7}\) copies g\(^{-1}\), and rose to 6.92 \(10^{10}\) and 9.21 \(10^{9}\) copies g\(^{-1}\) on day 18 p.i. in CR1 and CR2, respectively (Fig. 5). Rat HEV RNA was detected at day 21 p.i. in sera of CR1 and CR2 at 8.50 \(10^{5}\) and 9.20 \(10^{5}\) copies ml\(^{-1}\), respectively.
Genome sequence comparison of rat HEV recovered from nude rats and cell culture

The entire genome sequences of rat HEV recovered from nude rat faeces (sample taken from LR1 on day 30 p.i.) and cell culture supernatant (sample collected from day 68 p.i. of the first passage) were analysed by next-generation sequence analysis and compared with the sequence of the cDNA clone. The identified mutations and the deduced amino acid exchanges are shown in Table 1. The genome sequences of the cDNA clone and the rat HEV recovered from the intrahepatically inoculated nude rat were identical. In contrast, nine mutations were found in rat HEV recovered from the cell culture, which resulted in four
synonymous and five non-synonymous mutations in ORF1 and ORF2. The mutations at positions 295 and 343 were synonymous for ORF1, but non-synonymous for the overlapping ORF4.

**DISCUSSION**

Rat HEV is a recently discovered virus related to, but distinct from, human HEV (Johne *et al.*, 2014a). As laboratory rats can be reproducibly infected with rat HEV and a cell culture propagation system is available for rat HEV, this virus may be used in the future as a surrogate virus for human HEV, enabling studies on various aspects of virus replication and the immune response. However, a system for genetic manipulation of rat HEV has been missing. The recent publication of a cell culture system for rat HEV (Jirintai *et al.*, 2014) prompted us to analyse the infectivity of the generated rat HEV for cell culture. The results of the experiments showed that the virus replicated – after a lag phase of 30 days – with high titres in PLC/PRF/5 cells. After a passage in this cell culture system, virus growth was observed earlier and with even higher titres. Notably, the obtained titres of >10⁹ genome copies ml⁻¹ are considerably higher than those described for other rat HEV strains [10⁷ genome copies ml⁻¹ (Jirintai *et al.*, 2014)] or for human HEV strains [10⁷ genome copies ml⁻¹ (Okamoto, 2011)].

| Genome analysis identified several point mutations in the recovered virus, which may be linked to cell culture adaptation. Although infectivity of the recovered virus for nude rats could still be demonstrated, the biological significance of these mutations has to be assessed in future studies. Direct transfection experiments of cell cultures with in vitro transcribed RNA of the original clone compared with a clone containing the mutations may be performed in order to assess the significance of the mutations for efficient cell culture growth. A direct transfection system would generally be useful for cell culture studies on rat HEV without the need for nude rat inoculation.

In summary, the generation of a versatile reverse genetics system for rat HEV has been shown here. After introduction of specific mutations into the cloned cDNA, the system may be useful to study the phenotypic effects of the mutations. As rats are widely used and well-characterized
laboratory animals, studies on genetically engineered rat HEV may provide novel insights into organ tropism, replication and excretion kinetics as well as immunological changes induced by hepeviruses.

**METHODS**

**Generation of a genomic clone of rat HEV.** The complete genome of rat HEV was amplified in four fragments by RT-PCR using RNA isolated from the liver of a wild rat from Germany containing the rat HEV prototype strain R63/DEU/2009 (GenBank accession number GU345042). RT-PCR was performed with a LongRange 2Step RT-PCR kit (Qiagen) and primers listed in Table S2. The primer binding sites overlapped with unique binding sites for restriction enzymes within the rat HEV genome, thus enabling subsequent cloning of the RT-PCR products. The 5′ end primer contained a T7 RNA polymerase promoter sequence and the 3′ end primer contained an extension of 17 thymidine residues followed by a unique XbaI site (Fig. 1a). The RT-PCR products were cloned successively into a pUC19 vector derivative, which contained a multiple cloning site adapted to the restriction sites used. The sequence of the resulting genomic clone was determined by Sanger sequencing using the PCR primers and additional primers.

**In vitro transcription for the generation of capped RNA.** The genomic plasmid was purified using a Plasmid Maxi kit (Qiagen) and subsequently linearized with XbaI. The preparation was further purified by phenol/chloroform extraction and resuspended in RNase-free water. *In vitro* transcription was performed using a mMESSAGE mACHINE T7 kit (Ambion), which included adding a cap analogue to the 5′ end of the synthesized RNA, according to the protocol of the supplier. RNA purification was carried out by lithium chloride precipitation.

**Inoculation of rats and sample collection.** Eight nude rats (Long-Evans *rnu/rnu*; Japan SLC) and two specific-pathogen-free rats (Wistar; Japan SLC) were used in this study. The rats were individually housed in Biosafety Level 2 facilities. All rats were negative for rat HEV RNA and anti-rat HEV antibodies, as determined by nested broad-spectrum RT-PCR (Johne et al., 2010a) and rat HEV-specific ELISA (Li et al., 2013b), respectively. To analyse the infectivity of the *in vitro* transcribed RNA, intravenous injection and intrahepatic inoculation were carried out. The RNA (2.6 × 10⁹ copies µl⁻¹) was injected through the tail vein or through a percutaneous inoculation procedure into five different sites of the liver with ~100 µl per injection site. The rats were injected under xylazine/ketamine hydrochloride anaesthesia. To examine the infectivity of recovered rat HEV, stool specimens were diluted in PBS to prepare 10% suspensions by shaking at 4°C for 1 h. Both the cell culture supernatants and 10% stool suspensions were clarified by centrifugation at 10 000 g for 30 min, and then passed through a 0.45 µm membrane filter (Millipore). The rats were intravenously inoculated with the stool suspension or with cell culture supernatant through the tail vein. Serum samples from the nude rats were collected at the end of the experiment for detection of rat HEV RNA. Serum samples from the Wistar rats were collected weekly for examination of rat HEV-specific IgG antibodies and ALT values. Stool samples were collected one to two times per week. The experiments were reviewed by the ethics committee of animal welfare, National Institute of Infectious Diseases, and carried out according to the ‘Guides for Animal Experiments Performed at the National Institute of Infectious Diseases’ under codes 113029 and 114012.

**Inoculation of cell culture.** The human hepatocarcinoma cell line PLC/PRF/5 (JCRB0406) from the Health Science Research Resources Bank (Osaka, Japan) was used. Cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% (v/v) heat-inactivated FBS (Nichirei), 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (Gibco) at 37°C in a humidified 5% CO₂ atmosphere. For virus inoculation, confluent cells were trypsinized, diluted 1:3 and cultured in a 25 cm² tissue culture flask. On the next day, the medium was removed and the cells were washed with PBS. A total of 1 ml sample was inoculated onto PLC/PRF/5 cells. After adsorption at 37°C for 1 h and washing of the cells two times with PBS, the suspension was removed and then replaced by 10 ml maintenance medium consisting of medium 199 (Invitrogen) containing 2% (v/v) heat-inactivated FBS and 10 mM MgCl₂. Further incubation was at 36°C. The culture maintenance medium was replaced with new medium every 4 days, and used for the detection of rat HEV RNA and capsid antigen. Cells were observed daily by light microscopy for the occurrence of a cytopathic effect.

**Quantitative real-time RT-PCR for detection of rat HEV.** The RNA was extracted using the MagNA Pre LC system with a MagNA Pre LC Total Nucleic Acid isolation kit (Roche Applied Science).
Detection of anti-rat HEV IgG and rat HEV capsid antigen. Anti-rat HEV IgG was detected by ELISA as described previously (Li et al., 2011). An antigen capture ELISA was used to detect rat HEV antigen. Briefly, duplicate wells of flat-bottom 96-well polystyrene microplates (Dynex) were coated with 100 µl coating buffer (0.1 M carbonate/bicarbonate buffer, pH 9.6) containing 1:1000 dilution of a hyperimmune serum elicited in a rabbit with rat HEV-like particles (Li et al., 2011). The coating was performed at 4 °C overnight. Unbound antibodies were removed and the wells were washed twice with 10 mM PBS containing 0.05% Tween 20 (PBS-T), and then the blocking was carried out at 37 °C for 1 h with 150 µl 5% skim milk (Difco) in PBS-T. Aliquots of 100 µl cell culture supernatants were added to the wells and incubated for 1 h at 37 °C. After the wells were washed three times with PBS-T, 100 µl guinea pig rat HEV-like particle hyperimmune serum (1:1000 dilution with PBS-T containing 1% skim milk) was added to the wells and the plate was incubated for 1 h at 37 °C. The plate was washed three times with PBS-T and then HRP-conjugated goat anti-guinea pig IgG antibody (Cappel) (1:1000 in PBS-T containing 1% skim milk) was added to each well. After incubation for 1 h at 37 °C, the plate was washed three times with PBS-T, and 100 µl substrate O-phenylenediamine and H2O2 was added. The plate was left for 30 min at room temperature and then the reaction was stopped with 50 µl 4 N H2SO4. A492 was measured with a microplate reader (Molecular Devices). The cut-off was defined using supernatants of non-infected cell cultures and set as 0.150. Test samples were considered positive when the A492 was above the cut-off value.

Viral genome sequencing. The whole-genome sequence of rat HEV present in selected samples was analysed by next-generation sequencing. Virus particles were enriched from the samples by caesium chloride density-gradient centrifugation (Li et al., 2011). A 200 bp fragment library was constructed for each sample preparation using a NEBNext Ultra RNA Library Prep kit for Illumina version 2.0 (New England Biolabs) according to the manufacturer’s instructions. Samples were bar-coded for multiplexing using NEBNext Multiplex Oligos for Illumina, Index Primer Sets 1 and 2 (New England Biolabs). Library purification was done using Agencourt AMPure XP magnetic beads (Beckman Coulter) as recommended in the NEBNext protocol. The quality of the purified libraries was assessed on a MultiNA MCE-915 (Shimadzu) and the concentrations were determined on a Qubit 2.0 fluorometer using the Qubit HS DNA assay (Invitrogen). A 151-cycle paired-end read sequencing run was carried out on a MiSeq desktop sequencer (Illumina) using MiSeq Reagent kit version 2 (300 cycles). Following preliminary analysis, the MiSeq reporter programme was used to generate FASTQ formatted sequence data for each sample. Sequence data were analysed using CLC Genomics Workbench Software version 7.5.1 (CLC Bio). Contigs were assembled from the obtained sequence reads by de novo assembly. Missing sequences of the 5’-terminal non-coding regions of the genomes were determined using the a Rapid Amplification of cDNA Ends kit (Invitrogen) according to the manufacturer’s instructions. In order to identify point mutations, the generated sequences were compared with that derived from the cDNA clone using DNAsis-Mac version 3.0 (Hitachi Solutions).

Liver enzyme level. ALT values in rat sera were monitored weekly by a Fuji Dri-Chem Slide GPT/ALT-P1II kit (Fujiﬁlm). The geometric mean of ALT values during the pre-inoculation period of each animal was deﬁned as the normal ALT value and a twofold or greater increase at the peak was considered as a sign of hepatitis.

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